Effect of Cell Origin and Timing of Delivery for Stem Cell-Based Bone Tissue Engineering Using Biologically Functionalized Hydrogels

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Despite progress in bone tissue engineering, the healing of critically sized diaphyseal defects remains a clinical challenge. A stem cell-based approach is an attractive alternative to current treatment techniques. The objective of this study was to examine the ability of adult stem cells to enhance bone formation when co-delivered with the osteoinductive factor bone morphogenetic protein-2 (BMP-2) in a biologically functionalized hydrogel. First, adipose and bone marrow-derived mesenchymal stem cells (ADSCs and BMMSCs) were screened for their potential to form bone when delivered in an RGD functionalized alginate hydrogel using a subcutaneous implant model. BMMSCs co-delivered with BMP-2 produced significantly more mineralized tissue compared with either ADSCs co-delivered with BMP-2 or acellular hydrogels containing BMP-2. Next, the ability of BMMSCs to heal a critically sized diaphyseal defect with a nonhealing dose of BMP-2 was tested using the alginate hydrogel as an injectable cell carrier. The effect of timing of therapeutic delivery on bone regeneration was also tested in the diaphyseal model. A 7 day delayed injection of the hydrogel into the defect site resulted in less mineralized tissue formation than immediate delivery of the hydrogel. By 12 weeks, BMMSC-loaded hydrogels produced significantly more bone than acellular constructs regardless of immediate or delayed treatment. For immediate delivery, bridging of defects treated with BMMSC-loaded hydrogels occurred at a rate of 75% compared with a 33% bridging rate for acellular-treated defects. No bridging was observed in any of the delayed delivery samples for any of the groups. Therefore, for this cell-based bone tissue engineering approach, immediate delivery of constructs leads to an overall enhanced healing response compared with delayed delivery techniques. Further, these studies demonstrate that co-delivery of adult stem cells, specifically BMMSCs, with BMP-2 enhances bone regeneration in a critically sized femoral segmental defect compared with acellular hydrogels containing BMP-2.

Introduction

IN INSTANCES OF VOLUMETRIC bone loss, such as in traumatic injury or in tumor resection, loss of progenitor cells or damage to surrounding tissue can limit the endogenous healing capacity of the patient, resulting in nonunion of the defect.¹ There is a large clinical need for effective treatment of such defects, as more than 500,000 bone grafting procedures occur annually, resulting in costs in excess of \$2.5 billion in the United States alone.² The clinical gold standard for treatment of large segmental bone defects is autologous bone grafting. This procedure, however, is severely constrained by a limited supply of available graft material and significant donor site morbidity.^{3,4} An alternative treatment is processed bone allografts. Again, this treatment

has significant limitations, including an unacceptably high rate of postimplantation failure, largely attributable to the inability of the graft tissue to fully revascularize and remodel.^{3,5} The occurrence of refracture in allograft treatment strategies varies depending on the size of the graft and other factors, but has been reported to be as high as 25–35%.⁶ There are additional concerns with regard to disease transmission and immune rejection with allograft use.^{3,7–9} As such, alternative treatment strategies are warranted to address the shortcomings of current treatment modalities.

A new grafting approach has emerged in the clinical treatment of diaphyseal defects: the Masquelet technique. Briefly, this is a multi-step procedure; in the first procedure, debridement of the bone and surrounding soft tissue followed by placement of a cement spacer in the area of a bone

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defect is performed.^{10–12} The spacer serves to both prevent fibrous tissue invasion into the defect site and induce the formation of a fibrotic capsule around the defect site. In the second procedure, the spacer is removed with minimal disruption of the newly formed membrane. Trabecular bone chips are placed in the defect site to facilitate bridging. This technique has been shown to be effective both in animal models and in clinical practice for tibial and femoral defects.^{13,14} The membrane surrounding the spacer has been shown to be well vascularized and has cells expressing angiogenic and osteogenic factors, including bone morphogenetic protein-2 (BMP-2), which is hypothesized to contribute to the healing of the defect.¹⁵

While the Masquelet technique has been shown to be effective in the treatment of long bone defects, it is still dependent on the use of autologous or allograft tissue. Utilizing stem cells and osteoinductive growth factors in a tissue engineering approach for bone regeneration may alleviate the need for grafting substrates. Previous studies have demonstrated that injectable alginate-based hydrogels are an effective strategy for growth factor delivery, specifically BMP-2, in the treatment of critically sized diaphyseal defects in a guided bone regeneration technique.^{16,17} The potency of BMPs results in large volumes of bone forming quickly, and the tissue may not be able to be maintained in the long term.¹⁸ As such, reducing the amount of BMP needed for restoration of a defect may lead to better clinical outcomes. One such way to reduce BMP dosage is via stem cell co-delivery. Several studies have evaluated bone tissue engineering using bone marrow and adipose-derived mesenchymal stem cells (BMMSCs and ADSCs).^{19–30} However, there have been few studies on the incorporation of stem cells into an alginate hydrogel delivery system, and timing of delivery of the alginate hydrogel to the defect site has yet to be investigated.^{31,32}

Previous studies in our lab have demonstrated a dose-dependent bridging rate and mechanical integrity with the amount of BMP-2 delivered to diaphyseal defects in the femur.¹⁷ The purpose of this study was to investigate the use of a similar injectable hydrogel system loaded with adult stem cells to heal a critically sized diaphyseal defect in the femur of the rat, and further, to test the effect of the delivery time of the hydrogel to the defect site. First, the feasibility of using alginate hydrogels as an injectable cell carrier by assessing cell viability and DNA content in the hydrogels *in vitro* was examined. Next, a screening of different cell types in a subcutaneous implant model to identify the most promising candidates for bone regeneration in an orthotopic site was performed. After determining a candidate from the subcutaneous screening results, the effect of timing of therapeutic delivery on the bone regeneration response was examined using a femoral segmental defect model. Potential delayed delivery time points were identified by examining tissue infiltration into empty femoral defects over a 14 day time course. For delayed delivery, a two-step surgery technique similar to the Masquelet technique previously described was employed. The second procedure at 7 days involved injecting the hydrogel with as little damage to surrounding tissues as possible. All experiments used a nonhealing dose of BMP-2 to assess the effect of the delivered adult stem cells on the repair process.

Materials and Methods

Isolation of GFP ADSCs and BMMSCs

Genetically modified Sprague–Dawley rats that express GFP ubiquitously (SD-Tg(GFP)2BalRrc) were obtained from the Rat Resource and Research Center (RRRC, Columbia, MO). Three males weighing 120–150 g were euthanized, and the adipose tissue and bone marrow were harvested as previously described.^{29,33} Briefly, adipose tissue was pooled, washed thrice in Hank's balanced saline solution (Life Technologies, Grand Island, NY), and digested in 0.25% trypsin (Life Technologies) for 30 min at 37°C. The tissue was then cut into smaller pieces and digested in 9125 units of collagenase IA (Sigma, St. Louis, MO) and 75 units of dispase (Gibco, Invitrogen, Carlsbad, CA) for 3 h. The upper layer of adipocytes was removed, and the cell suspension was filtered through a 40 µm cell strainer. The digestion was stopped with MSC growth media (GM) (Lonza, Basel, Switzerland), and the cells were collected by centrifugation. The cells were plated at 5000 cells/cm² in T-75 flasks. Cultures were washed twice with phosphate-buffered saline (Mediatech, Manassas, VA), fed with GM at 24 and 48 h after plating, and allowed to grow to 90% confluence. The cells were cryopreserved after expansion. Vials of cells were thawed and expanded by one passage to generate the cell numbers necessary for the experiments.

For BMMSCs, femora and tibiae were harvested aseptically. Both epiphyseal ends were removed, and the marrow was flushed out with α -Minimum Essential Medium (MEM) into tissue culture plates (Gibco). After 30 min, the media was collected and then re-plated onto new tissue culture plates. Forty eight hours later, the media was aspirated and the adherent cells were grown to 90% confluence, with media changes occurring every 48 h. The cells were then cryopreserved after expansion. Vials of cells were thawed and expanded by one passage to generate the cell numbers necessary for the experiments.

Alginate hydrogel preparation

Alginate hydrogels were prepared as previously described.¹⁶ Sterile irradiated RGD functionalized alginate was obtained from FMC Biopolymer (FMC Biopolymer, Sandvika, Norway). Alginate hydrogels were prepared by dissolving the lyophilized polymer in α -MEM to a 3% w/v solution. The 3% w/v solution was then diluted to a 2% solution with either α -MEM or α -MEM containing 1 million cells per 150 µL of hydrogel. For BMP-2 (R&D Systems, Minneapolis, MN) containing gels, rat serum albumin (RSA, Sigma Aldrich, St. Louis, MO) was dissolved in 4 mM hydrochloric acid to obtain a 0.1% solution and then mixed with the lyophilized BMP-2 protein at a 10 µg/100 µL concentration. The BMP-2 solution was added to a syringe to obtain a final concentration of 2 µg/150 µL of hydrogel for subcutaneous experiments, and 1 µg/150 µL of hydrogel for femoral bone regeneration experiments. All samples had 150 µL of hydrogel total corresponding to 2 µg per sample in subcutaneous implants, and 1 µg per sample in diaphyseal defects. The 1 µg dose for diaphyseal defects was chosen after evaluating the results from the subcutaneous model with the rationale that the 2 µg dose may overwhelm any response from implanted cells in an orthotopic site. Hydrogels lacking BMP-2

only received the RSA carrier solution. Alginate hydrogels were then cross-linked with a 0.21 g/mL calcium sulfate solution.

Cell viability and histomorphometric analysis

For *in vitro* analysis of cell viability in the alginate hydrogel, hydrogel was extruded into PCL nanofiber mesh tubes. PCL nanofiber mesh tubes were prepared and sterilized as previously described.^{16,34} Hydrogels were removed from culture and stained for dead cells using the ethidium homodimer-1. A 1:1000 dilution of the ethidium homodimer-1 was prepared in PBS as per the manufacturer's instructions (Invitrogen, Eugene, OR), and the hydrogels were placed in 2 mL of solution and agitated gently on a rocker plate for 1 h (Stovall Life Scientific, Greensboro, NC). Hydrogels were then removed from the PCL nanofiber mesh and placed in a confocal imaging chamber. For each hydrogel, three regions were randomly selected, imaged at 10 $\times$ magnification on a Zeiss Axio Observer (Zeiss, Gottingen, Germany), and imaged for GFP-positive cells (live) and ethidium homodimer-1 stained dead cells.

Alginate digestion and DNA quantification

Alginate hydrogels in their PCL nanofiber mesh tubes were placed in a 1.5 mL Eppendorf tube (Eppendorf, Hamburg, Germany) and immersed in 82.5 mM sodium citrate digestion solution. The tubes were placed in a water bath at 37°C for 5 min. The PCL mesh tube was then removed, and samples were spun at 15,000 rpm for 10 min. The digestion solution was then aspirated, leaving a cell pellet, and the cells were lysed with 0.05% Triton-X 100 in PBS, a 10 s sonication, and one freeze-thaw cycle. DNA was measured as per manufacturer's instructions using the Picogreen DNA quantification assay (Invitrogen). Data are presented as a percentage of the DNA levels immediately after hydrogel formation (Day 0 levels).

Subcutaneous ectopic mineralization model

Female RNU Nude rats approximately 14 weeks of age underwent a subcutaneous implant procedure (Harlan, Tampa, FL). Aseptic procedures were followed. Two 2 cm incisions were made on the dorsal side of the rat. Two pockets were made lateral to the incision sites by blunt dissection. A stainless steel tube containing the PCL nanofiber mesh with the hydrogel was inserted into the subcutaneous pockets, and the hydrogels were placed into individual pockets away from the incision site. Incisions were then closed with a subcutaneous suture and wound clips. The groups tested for *in vivo* mineralization are listed in Table 1 and are as follows: ADSCs co-delivered with BMP-2 in an alginate hydrogel (+ADSC/+BMP-2), an acellular alginate hydrogel containing BMP-2 (–SC/+BMP-2), BMMSCs co-delivered with BMP-2 in an alginate hydrogel (+BMMSC/+BMP-2), BMMSCs in an alginate hydrogel lacking BMP-2 (+BMMSC/–BMP-2), and a no-hydrogel group that consisted of BMMSCs and BMP-2 in α -MEM without an alginate hydrogel carrier (No Hydrogel+BMMSC/+BMP-2). The final group was implanted by placing the mesh tube in the subcutaneous pocket, then injecting the solution into the mesh tube. All procedures were approved by the Georgia Institute of Technology Institute Animal Care and Use Committee.

TABLE 1. SUBCUTANEOUS IMPLANT GROUPS

Group	RGD-alginate	2 μ g BMP-2	1 $\times$ 10 ⁶ cells
+ADSC/+BMP-2	+	+	+
–SC/+BMP-2	+	+	–
+BMMSC/+BMP-2	+	+	+
+BMMSC/–BMP-2	+	–	+
No hydrogel +BMMSC/+BMP-2	–	+	+

ADSCs, adipose-derived mesenchymal stem cells; BMMSC, bone marrow-derived mesenchymal stem cells; BMP, bone morphogenetic protein.

Diaphyseal defect procedures

Female RNU Nude Rats (Harlan) aged 14 weeks underwent a bilateral segmental defect procedure as previously described.^{17,35} Briefly, critical-sized diaphyseal defects were created in the femur. Defects were stabilized with a polysulfone internal fixation plate modified with two holes in the center to enable a hydrogel injection into the defect site. After attachment of the fixation plate, an 8 mm segment of bone was removed from the diaphysis of the femur. A PCL nanofiber mesh tube was then placed around the defect site. For the empty defect experiment and delayed defect groups, the defect site was left unfilled with the surrounding muscle sutured and the skin closed with wound clips. Groups receiving immediate treatment had a hydrogel injected into the defect site, followed by closure.

For animals receiving delayed implantations, the initial incision was reopened at 7 days post creation of the defect. The wound clips from the initial surgery were removed. Scabbed tissue was cut away from the initial incision using a scalpel, and the incision was reopened. The middle sutures in the surrounding muscle were cut, the syringe containing the hydrogel was then guided through the center holes of the fixation plate, and 150 μ L of hydrogel was delivered inside the nanofiber mesh. The muscle was then re-sutured around the fixation plate, and the skin was closed via wound clips. The groups tested for both immediate and delayed delivery in the diaphyseal defect model are outlined in Table 2. Briefly, a negative control showing the baseline response form the alginate material was tested. The addition of BMP-2 at 1 μ g per defect to the hydrogel and 1 million bone marrow MSCs with 1 μ g BMP-2 were evaluated for their bone regeneration potential.

Faxitron radiograph imaging and scoring

Digital radiographs (Faxitron MX-20 Digital; Faxitron X-ray Corp., Wheeling, IL) were performed at an exposure

TABLE 2. IMMEDIATE AND DELAYED DELIVERY GROUPS FOR DIAPHYSEAL DEFECT

Group	RGD-alginate	1 μ g BMP-2	1 $\times$ 10 ⁶ cells
–BMMSC/–BMP-2	+	–	–
–BMMSC/+BMP-2	+	+	–
+BMMSC/+BMP-2	+	+	+

time of 11 s and a voltage of 25 kV with the animals under anesthesia. Animals were imaged at weeks 4, 8, and 12 post-defect creation. Bridging rates were determined by blinded evaluation from three independent scorers.

Micro-CT imaging

Subcutaneous samples were explanted after 8 weeks, placed in neutral buffered formalin for 48 h, and then placed in PBS. Samples were placed in a custom holder for micro-CT scanning. Mineralized matrix composition of the constructs was determined using a VivaCT scanner (Scanco Medical, Brütisellen, Switzerland) with a 38.0 μm voxel size at 55 kVp, 109 μA , 1024 μs scaling, and a 200 ms integration time. The constructs were evaluated with a lower threshold of 80 with a Gaussian filter width of 1.2 and a filter support of 1.0. The total volume of the mineralized matrix was determined.

Live animal *in vivo* micro-CT scans (VivaCT 40; Scanco Medical) were performed with a 38.0 μm voxel size at a voltage of 55 kVp and a current of 109 μA . Analysis of newly formed bone was achieved with a lower threshold of 125, corresponding to approximately half the threshold of native cortical bone as previously used.^{17,36–39} This corresponds to a mineral density of approximately 380 mgHA/cm³. The scans were segmented such that only bone inside the nanofiber mesh was included in the analysis. Scans were performed at 4, 8, and 12 weeks post-defect creation.

Histological analysis

Samples were decalcified in Immunocal (Decal Chemical Corporation, Tallman, NY) and prepared for paraffin processing. Histological analysis was performed using H&E staining for general morphology, and Safranin-O for cartilage/bone tissue differentiation. For the empty defect characterization, immunohistochemistry was performed for total macrophage presence in decalcified empty defects via CD68 staining (AbD Serotec, Bio-Rad, Raleigh, NC). Subcutaneous and diaphyseal bone defect samples were stained

for the presence of GFP using a primary GFP antibody (Abcam, Cambridge, MA). Briefly, the immunohistochemistry procedure was as follows: Sections were permeabilized with 0.1% Triton-X 100 solution in PBS, blocked with 5% donkey serum, and stained with a 1:100 solution of primary antibody in PBS incubated overnight at 4°C. After washing, a secondary Texas Red fluorescently tagged antibody (Invitrogen) was incubated for 1 h at RT. Samples were cover slipped and imaged at 20 $\times$ magnification using the Zeiss Axio Observer. Sections were counterstained for nuclei using DAPI (Invitrogen) to discern antibody tagged cells from all cells.

Statistical analysis

Data are presented as the mean with standard error. Data were analyzed via ANOVA with a Tukey's *post-hoc* test for significance using GraphPad software. Significance was determined with an alpha level of 0.05.

Results

Cell viability in alginate hydrogels

The majority of the cells survived the mixing and injection process involved in generating the hydrogel as indicated by positive GFP signal and low ethidium homodimer-1 signal (Fig. 1A). Cell proliferation was confirmed with the Picogreen DNA assay, as a significant increase in the DNA content of the hydrogel constructs between days 1 and 7 was observed for both ADSC- and BMMSC-loaded hydrogels. The DNA content of ADSC hydrogels was significantly higher than BMMSC-loaded hydrogels at days 7 and 14 (Fig. 1B).

Subcutaneous ectopic mineralization in alginate hydrogels

The BMMSC (+BMMSC/+BMP-2) and acellular (–SC/+BMP-2) hydrogel groups produced the most ectopic mineralization *in vivo* as shown in the 3-D reconstructions

FIG. 1. (A) Representative LIVE/DEAD images of adipose and bone marrow-derived mesenchymal stem cells (ADSCs and BMMSCs) in injected alginate over a 14 day time course *in vitro*. (B) DNA content in alginate hydrogels. * $p < 0.05$ versus Day 0 levels for that cell type; \$ $p < 0.05$ for cell type at that time point. Scale bar = 200 μm . Color images available online at www.liebertpub.com/tea

(Fig. 2A). Micro-CT processing and quantification showed that the ADSC (+ADSC/+BMP-2) hydrogels had significantly less mineralization relative to the acellular -SC/+BMP-2 control (Fig. 2B). All six samples of the +BMMSC/+BMP-2 produced robust mineralized tissue. This group had significantly higher mineral volume than all other groups. Removal of either the alginate hydrogel carrier (No Hydrogel+BMMSC/+BMP-2) or the BMP-2 (+BMMSC/-BMP-2) resulted in a significant decrease in ectopic mineralization compared with the +BMMSC/+BMP-2 group. Figure 2C shows that the only the +BMMSC/+BMP-2 group had consistent, robust mineralization.

Histological analysis of GFP cells in subcutaneous implants

After micro-CT scanning, the presence of implanted GFP-positive cells was tested via immunohistochemistry. GFP-positive cells were found in cell-loaded constructs with hydrogels, but were not found in acellular constructs or constructs lacking hydrogels. The presence of cells was observed in acellular constructs demonstrating host cell invasion into the hydrogel (Fig. 3). The majority of the DAPI labeled cells present were not positive for GFP, demon-

strating that most tissue present in the construct after 8 weeks was the result of host cell invasion.

Empty defect characterization

For these experiments, an 8 mm segmental defect was created in the femur and a poly-caprolactone nanofiber mesh was implanted around the defect site with the interior left empty. Histological analysis showed that over the 14 day time course, there was a gradual filling of the defect site with granulation tissue (Fig. 4). At day 3, the defect site was still largely empty as indicated by H&E staining (Fig. 4A–D). Immunohistochemistry for CD68, a pan-macrophage marker, showed a few macrophages present at the defect site (Fig. 5A). Polymorphonuclear cells, indicative of neutrophils, were observed in the defect site and at the bone ends via H&E staining. At day 7, more tissue had accumulated in the defect site (Fig. 4E). CD68-positive cells in the defect site were present at this time point, demonstrating macrophage infiltration (Fig. 5B). A large polymorphonuclear cell presence was also observed via H&E staining (Fig. 4F–H). By day 14, the defect site was nearly completely filled with host tissue (Fig. 4I). Tissue adjacent to the native bone ends had an organized structure (Fig. 4J, L). There were also large numbers of macrophages and polymorphonuclear cells

FIG. 4. H&E stains of empty defects at 3 (A), 7 (E), and 14 days (I). Tissue morphology in the proximal (B, F, J), medial (C, G, K), and distal (D, H, L) defect sites. Scale bar = 100 μ m. H&E, hematoxylin and eosin. Color images available online at www.liebertpub.com/tea

present in the defect site at the 14 day time point (Figs. 4I–L and 5C).

Immediate versus delayed delivery effect on bone formation

At 14 days postsurgery, there was limited space for hydrogel delivery due to infiltrated tissue; so, the hydrogel was injected at the 7 day time point. Figure 6 shows representative radiographs and micro-CT reconstructions for the groups listed in Table 2 at 4, 8, and 12 weeks. At 4 weeks, *in vivo* micro-CT showed that there was little bone in the defect site; no differences were observed between groups, and no effect of timing of delivery was detected (Fig. 7A). *In vivo* micro-CT analysis at 8 weeks demonstrated that little to no bone formation occurred in the alginate hydrogels lacking BMMSCs and BMP-2 (–BMMSC/–BMP-2) (Fig. 7B). In the immediate delivery samples, the addition of BMP-2 (–BMMSC/+BMP-2) caused a significant increase in the bone volume compared with the –BMMSC/–BMP-2 group. Incorporation of BMMSCs (+BMMSC/+BMP-2) resulted in significantly higher bone volume at 8 weeks for immediate delivery samples; this group was significantly greater than the –BMMSC/+BMP-2 and –BMMSC/–BMP-2 hydrogels, as well as the delayed +BMMSC/+BMP-2 implantation group. Delayed therapeutic implantation resulted in significantly less new

bone formation in the –BMMSC/+BMP-2 hydrogels and the +BMMSC/+BMP-2 hydrogels compared with their immediate delivery counterparts. There were no significant differences observed between any of the groups for the delayed therapeutic delivery, and none of the defects were bridged by this time point.

Similar results were observed at 12 weeks, albeit with elevated levels of bone volume compared with 8 weeks (Fig. 7C). Significantly more bone was observed in –BMMSC/+BMP-2 hydrogels over –BMMSC/–BMP-2 hydrogels. The immediately delivered +BMMSC/+BMP-2 hydrogel had significantly greater bone volume than –BMMSC/+BMP-2 and –BMMSC/–BMP-2 hydrogels. For the delayed delivery groups, the +BMMSC/+BMP-2 group produced significantly greater bone tissue compared with the –BMMSC/–BMP-2 group. However, the delayed +BMMSC/+BMP-2 group still had significantly less mineral than its immediately delivered counterpart. Bridging rates for the groups tested are displayed in Figure 6D and show that only the immediate delivery samples had fully bridged defects as determined by blinded scores of radiographs. For the immediate delivery samples, bridging of the defects in +BMMSC/+BMP-2 samples occurred at a rate of 75% compared with a 33% bridging rate for –BMMSC/+BMP-2 defects. No bridging was observed in any of the delayed delivery samples for any of the groups.

FIG. 5. CD68 and DAPI immunohistochemistry staining for macrophage presence in the empty defects at 3 (A), 7 (B), and 14 days (C). Scale bar = 100 μ m. Color images available online at www.liebertpub.com/tea

FIG. 6. Representative radiograph and *in vivo* micro-CT reconstructions of diaphyseal defects for the -BMMSC/-BMP-2 (A), -BMMSC/+BMP-2 (B), and +BMMSC/+BMP-2 groups (C) at 4, 8, and 12 weeks. (D) Bridging rates as determined by three independent scorers. Color images available online at www.liebertpub.com/tea

Histological analysis

H&E and Safranin-O staining of -BMMSC/-BMP-2 hydrogel samples showed the presence of fibrous tissue along with intact alginate hydrogel (Fig. 8). No areas of mineralization were observed in the defect site for both the immediate and delayed samples for this group. For -BMMSC/+BMP-2 and +BMMSC/+BMP-2 containing hydrogels, areas of lamellar-like bone were observed. For both immediate and delayed samples, areas of alginate hydrogel were still ob-

served after 12 weeks. Interestingly, in +BMMSC/+BMP-2 alginate hydrogels, the remaining hydrogel showed areas of counterstain indicative of implanted cells while acellular hydrogels lacked areas of counterstain (arrows in Fig. 8). Most of these areas, however, did not stain for intact nuclei on H&E, suggesting the cells were no longer functional after 12 weeks. GFP signal was not detected after 12 weeks.

Discussion

The objective of this study was to examine the co-delivery of adult stem cells with BMP-2 in an injectable hydrogel system to produce bone tissue. Both a subcutaneous implant model and a critically sized diaphyseal defect were employed to discern differences in regenerative potential based on cell origin, the need for osteoinductive factor co-delivery, as well as the need for a hydrogel carrier to facilitate localized bone tissue production. The effect of delivery time on the bone regeneration response was also evaluated. A comparison of the capacities of ADSCs and BMMSCs to form mineralized tissue in alginate hydrogels in a subcutaneous implant model demonstrated that BMMSCs consistently produced robust mineralization while ADSCs did not. Characterization of empty defects to identify possible delayed delivery time points of the hydrogel to the defect demonstrated that by 7 days there was tissue infiltration but no total filling of the defect. By day 14, the defect was nearly completely filled with host tissue. A delivery approach based on the Masquelet technique was performed, by which the defect site was re-entered through the initial incision and the therapeutic was delivered. A detrimental effect of delayed implantation compared with immediate implantation was observed. However, a beneficial effect of stem cell delivery was observed compared with hydrogels lacking implanted cells with both delivery methods at 8 and 12 weeks.

Differences due to cell origin were observed in the alginate hydrogels. The DNA content of ADSC-loaded hydrogels was significantly higher than BMMSC hydrogels at days 7 and 14, consistent with previous reports of ADSCs

FIG. 7. Quantification of defect bone volume at 4 (A), 8 (B), and 12 weeks (C). * $p < 0.05$ versus -BMMSC/-BMP-2 for immediate groups; \$ $p < 0.05$ versus delayed implantation for that group, # $p < 0.05$ versus -BMMSC/+BMP-2 for immediate groups. Color images available online at www.liebertpub.com/tea

FIG. 8. H&E and Safranin-O stains of diaphyseal defects after 12 weeks. *Arrows* indicate areas of counterstain in the alginate where implanted cells were likely present. Scale bar=100 μ m. Color images available online at www.liebertpub.com/tea

being more proliferative than BMMSCs.^{21,40,41} The difference in *in vivo* bone formation between the ADSCs and BMMSCs may be attributable to two factors: cytokine signaling to host cells and the timing of host cell invasion. Histological analysis of the subcutaneous samples demonstrated a large host cell invasion into the hydrogels. In the case of adipose-derived stem cells, we hypothesize that waste products from the incorporated cells degrade the BMP-2 present in the hydrogel, preventing a host cell response to the osteoinductive factor. Further, the BMP-2 dose chosen in this study may have been too low to induce cytokine activity from the ADSCs reducing endogenous osteoprogenitor recruitment. Bone regeneration in a calvarial defect with adipose-derived stem cells treated with BMP-2 at a higher dose on a gel foam scaffold has been reported.⁴² Others have shown that additional factors besides BMP-2 are necessary to induce osteogenic differentiation of ADSCs.⁴³ As such, in this system, the ADSCs may have two detrimental effects on bone formation: too low of an osteoinductive factor signal to stimulate implanted cell differentiation, and degradation of the delivered growth factor preventing endogenous cell-mediated bone formation as observed with acellular hydrogels. For BMMSCs, we hypothesize that the BMP-2 is causing an upregulation of osteoinductive factor signaling by the implanted cells, resulting in constructs that produce consistent robust mineralization compared with acellular constructs. This may have ultimately led to greater endogenous osteoprogenitor cell recruitment, resulting in a greater mineralized tissue volume. Early time point studies examining the gene and

protein expression of ADSCs and BMMSCs in these systems are needed to evaluate these hypotheses.

A delayed delivery technique of progenitor cells has not been performed for orthopedic applications. Limited studies have been performed using delayed progenitor cell delivery for neural tissue regeneration via restoration of the vascular supply in the brain.^{44,45} These studies tried to indirectly regenerate neural tissue by restoring the vascular supply in the brain with progenitor cells. The rationale for investigation into the timing of delivery of mesenchymal stem cells originated from studies on the regeneration of cardiac muscle tissue after myocardial infarction. Several clinical studies have investigated the use of mesenchymal stem cells to repair damaged muscle tissue after myocardial infarct.^{46–50} Given the delicate state of patients after a myocardial infarction, researchers sought to determine whether harvesting of autologous mesenchymal stem cells at 2–3 weeks after the infarct and injecting them into the left ventricular wall would have a benefit on heart function.⁵⁰ It was reported that delivery of 150 million autologous MSCs resulted in no added benefit in terms of ejection volumes from the left ventricular lobe.^{49,50} However, others have had success in delivering stem cells to patients 5–7 days post infarct and seeing improvements in heart function.^{47,48} These studies show that timing of delivery is critical in order to achieve a therapeutic effect.

The importance of endogenous cells from the periosteum and surrounding tissue in segmental defect repair has been demonstrated.^{37,51,52} The time point chosen in our delayed delivery study was selected based on the assumption that the presence of endogenous tissue in the defect site may facilitate nutrient and waste exchange of the delivered stem cells

and facilitate recruitment of host progenitors before the degradation of BMP-2. However, given our results showing significantly less mineralized tissue in delayed samples compared with their immediate counterparts, our delivery time may have been too late. The granular tissue in the defect site may have inhibited bone formation, and the BMP-2 may have been degraded by inflammatory cells identified in the empty defect studies before recruitment of endogenous osteoprogenitor cells occurred. An alternative hypothesis is that immediate delivery of osteoinductive factors significantly affects the amount of endogenous cells recruited to the defect site. Finally, the duration of implanted cell survival may be a critical factor in the effectiveness of cell-based tissue engineering strategies. GFP signal was not detected after 12 weeks, suggesting the implanted cells were no longer alive at this time point. Assessing *in vivo* cell viability over time is a current active area of research in these models.⁵³ Elucidating the mechanisms underlying our results requires further study.

As previously stated, we adapted a form of the Masquelet technique in order to obviate the need for graft materials by delivering biologics. The Masquelet technique calls for a cement block in the defect site to prevent fibrous tissue invasion. Our membrane was perforated, enabling tissue invasion. We hypothesized that allowing some tissue infiltration into the defect would have a beneficial effect on our delivered biologics, thus leading to an increase in bone formation. The increased bone formation would result from tissue infiltration occurring between the creation of a defect and the delivery of a therapeutic, enabling greater nutrient exchange and a possible increase in the recruitment of endogenous osteoprogenitors to the defect site. However, the existence of an inflammatory infiltrate directed more at fibrous tissue formation than mineralized bridging in the absence of sufficient osteoinductive signals to attract progenitors to the defect may have caused the limited bone formation with delayed cell delivery. An additional comparison group with debridement of the defect region, including removal of some of the early granulation tissue, to prevent a fibrotic or nonregenerative bias, may have determined the effect of the granular tissue in the defect site on the delivered BMP-2 as well as any effect on delivered cell viability.

In conclusion, the superiority of BMMSCs in producing robust mineralized tissue compared with ADSCs was demonstrated using low-dose BMP-2 co-delivery in a subcutaneous implant model. When applied to a diaphyseal defect in the femur, delivery of BMMSCs resulted in greater regenerated tissue volume as well as a higher bridging rate compared with acellular controls. Finally, the effect of timing of delivery was examined. Constructs delivered at the time of injury showed greater restored tissue volumes than those of the delayed delivery constructs.

Disclosure Statement

No competing financial interests exist.

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